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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Examination of the enzyme structure for acetylcholinesterase (AChE) reveals two sites of ligand interaction: The *peripheral site* (P-site) located at the entrance of the gorge, and the *acylation site* (A-site) at the base of the gorge. Our goal is to develop high affinity cyclic peptide ligands specific for the P-site while allowing the passage of acetylcholine to the A-site for use by personnel at risk for nerve gas exposure. Our strategy involves the covalent tethering of cyclic inhibitors via a methanethiosulfonate (MTS) linkage to a cysteine on the AChE mutant, H287C. To validate this approach we used cationic ligands with demonstrated affinity for the AChE A-site that are attached to MTS tethers of various lengths. After labeling we separated modified from unmodified enzymes using affinity chromatography on acridinium resin. Enzymes modified with ligands having a significant effect on the A-site elute in a NaCl wash, while unmodified enzyme elutes only with the AChE inhibitor decamethonium. We compared the substrate dependence of hydrolysis rates on the substrate concentration for unmodified H287C as well as 3 modified enzymes and found that 2 of the modified enzymes shifted the hydrolysis curve, with large increases in K_{app} and K_{ss}. Selective inhibition by propidium at the P-site and tacrine at the A-site measured the effect of labeling. We compared K_{I2} values and found that the shortest tethers had little effect on K_{I2}. Medium length tethers interfered with propidium binding to the P-site while K_{I2} values for tacrine inhibition increased progressively as the size of the tethered ligand increases.

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INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7), a member of the α/β hydrolase family of enzymes, is responsible for terminating nerve impulses at cholinergic synapses by hydrolyzing acetylcholine at one of the fastest known enzymatic rates (1). AChE crystal structures reveal a large active site gorge made up of two distinct sites connected by a narrow channel: the acylation site (A-site) at the base of the gorge and the peripheral site (P-site) at its mouth (2). Ligands can bind to either site exclusively or bridge both sites simultaneously. The P-site selectively binds inhibitors like propidium, a small aromatic compound, and fasciculin, a snake venom neurotoxic peptide.

One of our long-term goals is to utilize a better understanding of the role of the P-site to design P-site inhibitors that will selectively block the inactivation of AChE by organophosphate (OP) pesticides and nerve gas agents. Ligands that bind to the P-site inhibit AChE through a process we have called *steric blockade* (3,4). This process involves a decrease in the rate constants with which substrates and their hydrolysis products enter and exit the A-site. The concept of steric blockade has led to a new strategy for the design of drugs to protect AChE from OP inactivation. This strategy is to design cyclic compounds that will bind to the P-site and selectively block the access of OPs while allowing entry of acetylcholine. To initially localize prospective compounds to the P-site, we propose to tether them by disulfide linkage to a nearby cysteine residue. Wild type human AChE has no free cysteine residues, and we have introduced H287C near the P-site by site-specific mutagenesis. Prospective compounds are synthesized with a methanethiosulfonate (MTS) group, a highly reactive functional group that reacts with free cysteines to form a disulfide bond, thereby allowing their covalent linkage to H287C

AChE (5). In this report we test the feasibility of this approach using cationic trimethylammonium or acridinium ligands with demonstrated affinity for the AChE active site that are attached to MTS tethers of various lengths. We show that the length of the tether provides a molecular ruler which determines the catalytic properties of the modified AChEs.

BODY

We labeled H287C AChE with 6 different MTS-derivatized compounds (see Table 1 below) and evaluated their effectiveness in blocking the P-site. Some of these modified enzymes lost substantial activity, making it difficult to determine their catalytic properties in the presence of residual unmodified AChE. Therefore, to monitor AChE concentrations, we labeled the amine groups in H287C AChE by reductive methylation with [^{3}H]formaldehyde and sodium cyanoborohydride (6). Radiomethylation has no effect on AChE catalytic activity. We first found that the MTS-derivatized compounds did not react completely with H287C AChE and that we needed to separate the modified from the unmodified enzyme. This separation was achieved effectively by affinity chromatography on acridinium resin (7) if the modified enzyme had lost substantial catalytic activity. For example, unmodified AChE is retained on the affinity column during a wash with buffered 0.5 M NaCl and only is eluted when the AChE inhibitor decamethonium is introduced, as in Figure 1A. In contrast, AChEs modified with compounds III-VI were retained less tightly and eluted with 0.5 M NaCl (see Figure 1B). Modification with compounds I and II had less effect on the active site and resulted in AChEs which, like the unmodified AChE, required decamethonium for elution (Figure 1A). These compounds apparently do not extend far enough into the P-site to significantly alter high affinity binding by the acridinum resin. Comparison of the relative catalytic activity per enzyme site (enzyme activity/dpm) for the H287C AChE reaction with compound IV showed a much lower ratio for the modified enzyme that was eluted with NaCl than for the residual unmodified enzyme that was eluted with decamethonium (see Figure 1B).

To confirm such a change in relative activity, we next compared the dependence of hydrolysis rates v on the substrate concentration for the unmodified H287C AChE and the enzyme modified with compounds II, IV and VI. (Figure 2). The unmodified enzyme as well as enzyme modified with compound II showed the bell-shaped profiles typical of wild type AChE (see (4)), with substrate inhibition at high substrate concentrations. This result again indicates that the interaction of ligands and substrates with the AChE active site is relatively unaffected by modification with compound II. The hydrolysis profiles for H287C AChE modified with compounds IV and VI were shifted to the right in Figure 2, and no substrate inhibition was apparent following modification with compound VI. Analysis of these profiles with the Haldane equation for substrate inhibition confirmed that these modifications resulted in large increases in the values of the Michealis constant K_{app} and the substrate inhibition constant K_{SS} (8). Furthermore, the relative activity per enzyme site following modification with compound VI was at least 500-fold lower than that for the unmodified enzyme at low substrate concentrations. These observations indicate that access of acetylthiocholine to the acylation site was strongly blocked by modification with compound VI and moderately blocked by modification with compound IV.

To estimate the extent to which the tethered compounds blocked the P- and A-sites of H287C AChE, inhibition of enzyme activity by propidium and tacrine was compared. Propidium binds specifically to the P-site while tacrine binds specifically to the A-site, and the extent of their inhibition at low substrate concentrations provided an indication of their respective AChE affinities. However, as illustrated in Figure 2, contributions from residual unmodified enzyme activity were greatest at low substrate

concentrations, and quantitative analyses of the inhibition were required to take these contributions into account even after separation of the modified enzymes by affinity chromatography. The analyses employed equation 1, which assumes two populations of AChE characterized by different K_I values.

$$\frac{v_{I=0}}{v} \approx \frac{z_{I=0}}{z} = B \cdot \left[\frac{\left(1-R_2\right)\left(1+\frac{\alpha_1[I]}{K_{I1}}\right)}{\left(1+\frac{[I]}{K_{I1}}\right)} + \frac{R_2\left(1+\frac{\alpha_2[I]}{K_{I2}}\right)}{\left(1+\frac{[I]}{K_{I2}}\right)} \right]^{-1} \quad \text{(eq. 1)}$$

In equation 1 the second order hydrolysis rate constants in the presence (z) and absence ($z_{I=0}$) of inhibitor were approximated by the respective hydrolysis rates (v and $v_{I=0}$) at low acetythiocholine concentrations ([S] < $0.2K_{app}$) (see (9)). The K_Is are equilibrium dissociation constants for I with E, and the constants α are ratios of the second order rate constant with saturating I to that in the absence of I. Here K_{I1} and α_1 refer to the unmodified AChE population, K_{I2} and α_2 refer to the modified population, and R_2 is the fraction of total activity contributed by the modified population in the absence of inhibitor. The fitted parameter B reduced emphasis on the $v_{I=0}$ point and was 0.99 ± 0.06 for all analyses. Equation 1 was applied to data sets with 5 – 10 values of v obtained at various concentrations of inhibitor I. K_{I1}, α_1 and α_2 were fixed at the values fitted for the unmodified H287C AChE alone, and K_{I2}, R_2 and B were fitted simultaneously.

K_{I2} values obtained following modification of H287C AChE with compounds I – VI are listed in Table 1. These K_{I2} values are 2- to 95-fold larger than the K_{I1} value of unmodified H287C, and the magnitude of the increase corresponds to the extent to which

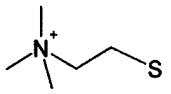
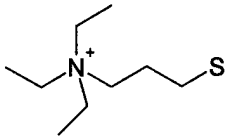
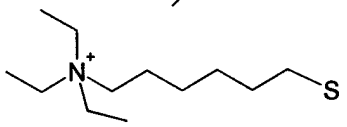
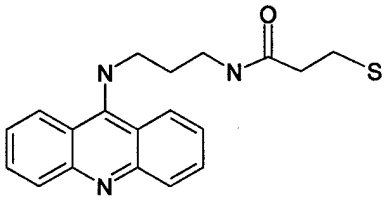
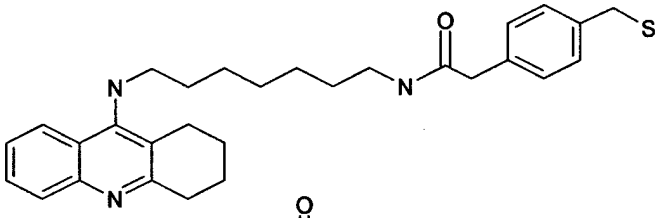
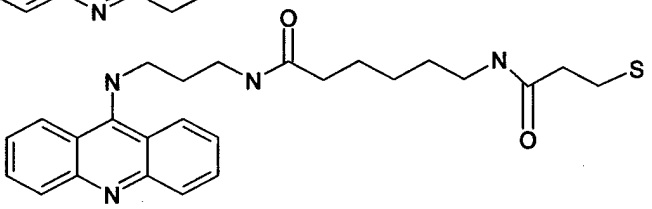
a given modification blocked access to the P-site or A-site. Modification with the shortest tethers in compounds I and II had little effect on K_{I2} values. AChEs modified with the medium length tethers in compounds III or IV were effective in interfering with propidium binding at the P-site. Increases in K_{I2} values for propidium of 30- to 95-fold were exhibited with these compounds, and significant further increases were not observed with the longer tethers in compounds V and VI. In contrast, K_{I2} values for tacrine inhibition increased only slightly following modification with compounds I - III and showed the greatest increase (90-fold) following modification with compound VI. Molecular modeling confirmed that compounds III and IV could reach the P-site but not extend into the A-site, while compounds V and VI could reach the A-site (8). These changes are summarized in the last column of Table 1, where the relative ratios of the tacrine affinity to the propidium affinity are listed. The relative ratios are similar to that of unmodified H287C AChE following modification with compounds I and II, where K_{I2} values showed little change, and with compounds V and VI, where K_{I2} values for both tacrine and propidium showed large increases of similar magnitudes. The relative ratios increased 8- to 14-fold following modification with compounds III and IV, where the tacrine affinity showed relatively little change but the propidium affinity fell significantly.

We are building on these observations in developing our strategy for the design of drugs to protect AChE from inactivation by OPs. Our search for a prototype of this drug has focused on cyclic peptide and pseudopeptide compounds, as they have a number of advantages. First, a cyclic molecule is a ring with a pore that in theory can be designed to prevent passage of bulky OPs to the A-site while interfering minimally with passage of

the smaller acetylcholine. Relatively small cyclic peptides consisting of 8 amino acids contain a pore size sufficient to permit passage of acetylcholine. Second, the incorporation of both natural and unnatural amino acids using combinatorial methods allows for synthesis of an enormous number of cyclic compounds in libraries of various size. Furthermore, cyclic peptides are conformationally constrained, an asset in molecular modeling studies. However, it is a challenge to identify a cyclic peptide with sufficient affinity for the AChE P-site to serve as a lead compound for further development. We developed MTS tethering as a transitional strategy to confine candidate peptide interactions to the close vicinity of the P-site. The site selected for tethering was H287, a residue on the active site gorge rim near the P-site but with a side chain that extends outward into solution, and its mutation to H287C introduced a unique site for modification by sulfhydryl reagents. The MTS class of sulfhydryl reagents has been useful in probing the acetylcholine binding sites in cysteine mutants of the nicotinic receptor (5). Furthermore, the sulfhydryl group in mouse H287C AChE was modified with compound I with little effect on the catalytic properties (10). We are now preparing cyclic peptides in which a lysine side chain is covalently linked to MTS, and these peptides will be tethered to H287C AChE. The modified enzymes will be screened for reduced affinity for acridinium resin as in Figure 1, and the catalytic activity of those with reduced affinity will be evaluated as shown in Figure 2 and Table 1. We anticipate that the enzyme surface accessible to the tethered peptide will be highly restricted. These restrictions should allow molecular modeling programs to generate more rigorous predictions of optimal peptide composition and configuration that result in maximal affinity for the entrance to the P-site. Experimental confirmation of these predictions

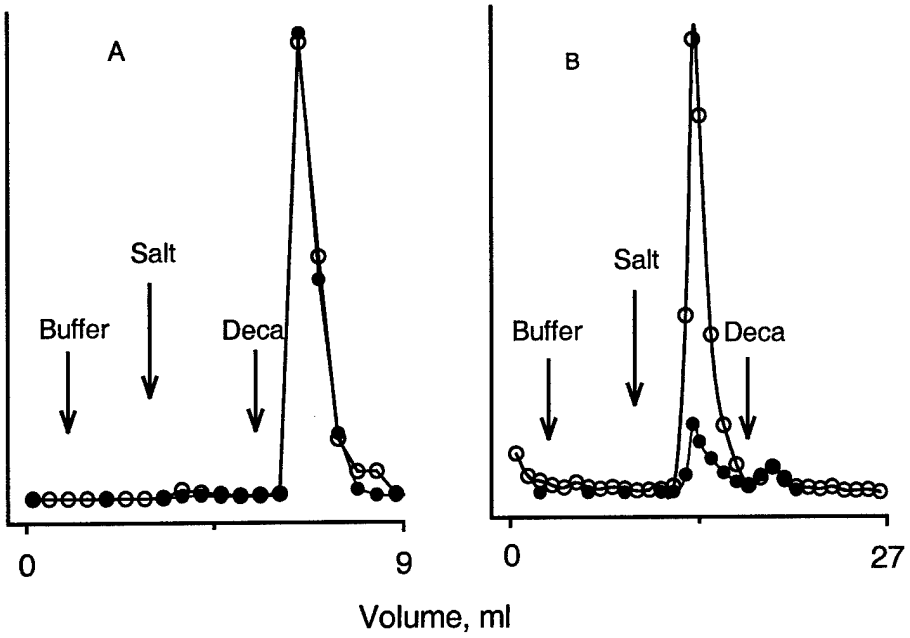
would then allow removal of the tethering constraint and demonstration of high peptide affinity for wild type AChE.

Table 1. Affinities of propidium and tacrine for H287C AChEs modified by MTS-derivatized compounds (8)[a]

Tethered Ligand		Propidium K_I [μM]	Tacrine K_I [nM]	Relative Ratio
Unmodified H287C		0.58	54	1.0
	I	2.1	160	1.2
	II	1.2	170	0.7
	III	16	110	14
	IV	54	670	8
	V	9.8	740	1.2
	VI	30	4800	0.6

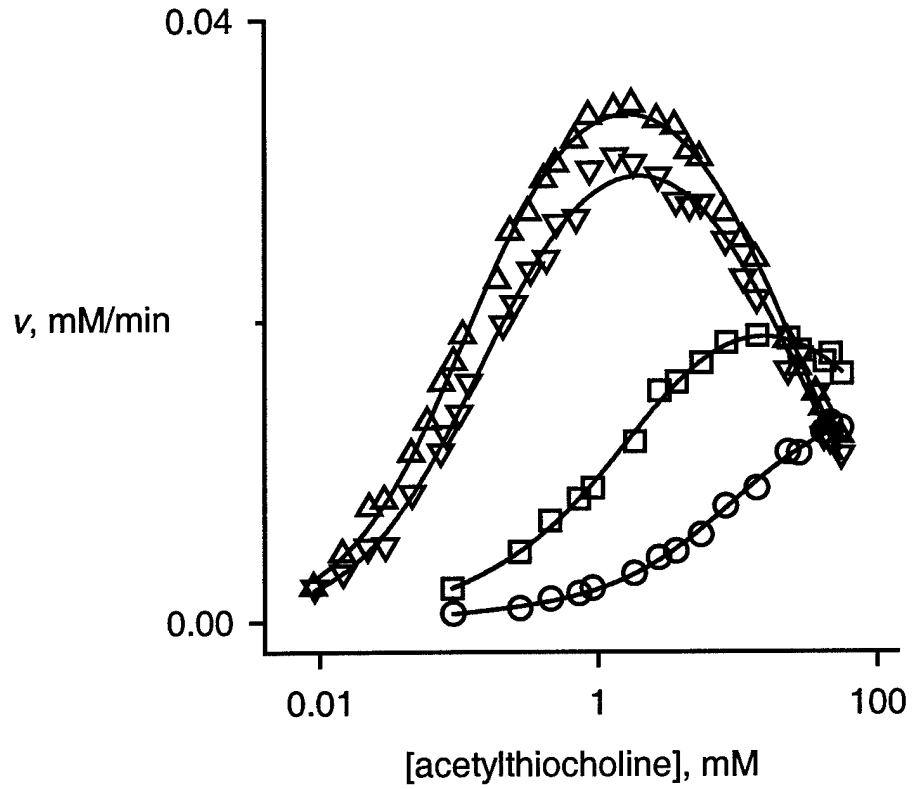
[a]For compounds I-VI, the indicated ligand is linked through the S atom to S-$(O)_2CH_3$ to give an MTS derivative. Following reaction with H287C AChE, the S atom is linked to the S of the enzyme cysteine. K_{I2} values were determined from equation 1. The "relative ratio" is defined as the ratio of the K_{I2} for propidium to the K_{I2} for tacrine for the modified AChE divided by the corresponding value for unmodified AChE.

Figure 1. *Acridinium resin affinity chromatography of H287C AChEs modified by MTS-derivatized compounds (8).*



H287C AChE was reductively radiomethylated with 10 mM [^{3}H]HCHO and 50 mM sodium cyanoborohydride (6) for 1 h. The dialyzed enzyme (3 - 12 μM) was treated with 1 mM dithiothreitol in 20 mM NaPi (pH 8.0) for 1 hour before addition of an MTS-derivatized compound (to 2 mM) for 30 min at room temperature. The reaction mixtures were dialyzed against 10 mM NaPi, pH 7.0 (buffer) and applied to an acridinium resin affinity column (1 ml, *panel A*; 5 ml, *panel B*). The column was washed sequentially with buffer, buffered 0.5 M NaCl, and buffered 0.5 M NaCl and 5 mM decamethonium bromide. Collected fractions were monitored by the spectrophotometric Ellman assay (see ref. 4) with 0.5 mM acetylthiocholine for enzyme activity (relative activity, -●-) and by liquid scintillation counting (relative dpm, -○-). *Panel A*: Enzyme modified with compound II. *Panel B:* Enzyme modified with compound IV. Relative y-axis scales were adjusted to give superposition of the activity and dpm values in the fractions eluted with decamethonium.

Figure 2. *Dependence of hydrolysis rates on the acetylthiocholine concentration for H287C AChEs modified by MTS-derivatized compounds (8).*



Hydrolysis rates v were measured at the indicated acetylthiocholine concentrations in 20 mM NaPi, 0.02% Triton X-100 (pH 7.0) at 25 °C. The ionic strength was maintained at a constant value by adding NaCl to fix the sum of the substrate and NaCl concentrations at 60 mM. The line for the unmodified AChE was calculated by fitting the data to the Haldane equation ($v = V_{max}[S]/(K_{app} + [S] + [S]^2/K_{SS})$. Lines for H287C modified with compounds II, IV and VI were calculated by fitting the data to the sum of two Haldane equations (8). Unmodified H287C AChE (△); H287C AChE modified with compounds II (▽); IV (□), and VI (○). The y-axis scale varied for each modified enzyme, and relative scales were chosen to clearly illustrate the shapes of the curves.

KEY RESEARCH ACCOMPLISHMENTS

- We have established a stable Schneider 2 *Drosophila* cell line expressing the H287C mutant AChE. This cell line is producing quantities of this enzyme to provide us with sufficient enzyme for the present and proposed studies.

- The development of procedures involving the labeling of H287C enzyme with MTS tethered compounds, has been worked out successfully. This method includes radiomethylation, followed by covalent linkage of MTS tethered compounds to the H287C. To date we have labeled H287C with 6 compounds successfully.

- After the labeling of H287C with MTS tethered compounds, we were confronted with the problem of separating the modified enzyme species from the unmodified. Using our knowledge of affinity chromatography, we have established a protocol for separating MTS modified enzymes that are affecting the affinity of the AChE peripheral site from unmodified enzyme.

- In experiments examining substrate dependence and hydrolysis, unmodified H287C and enzyme with tethered ligand II exhibit almost identical substrate dependence; ligand II cannot extend to the P-site or the A-site. For enzymes modified with ligand IV and VI, substrate dependence is shifted to the right which is consistent with dramatic increases in K_{app} and K_{SS}. These kinetic results fit well with the *steric blockade model*, that is, modifications IV and VI have dramatic effects on substrate entry into the A-site

- The effect of the tethered ligands blocking either the P-site or A-site was compared by testing inhibition with propidium and tacrine. Tethered ligands III and IV reach only to the P-site and selectively block propidium inhibition. Tacrine affinity for the A-site decreases progressively as the size of the tethered ligand increases, with the larger VI interfering significantly with access to the A-site.

- The results presented here with cationic trimethylammonium or acridinium ligands show that the length of the tether provides a molecular ruler which determines the catalytic properties of the modified AChEs thus providing for a proof of concept.

- This MTS tethering is a novel, transitional strategy we have developed to confine candidate peptide interactions to the close vicinity of the P-site. We are now synthesizing cyclic peptides with the MTS covalently linked through the lysine and will test these cyclic peptides using this strategy.

REPORTABLE OUTCOMES

PAPERS

Johnson, J.L., Cusack, B.M., Davies, M.P., Fauq A. and Rosenberry, T.L.. Substrate activation with a cationic acetanilide substrate in human acetylcholinesterase. *Biochemistry* in press.

Cusack, B.M., Johnson, J.L., Hughes, T.F., McCullough, E.H., Fauq, A., Romanovskis, P., Spatola, A.F. and Rosenberry, T.L.. Tethering of ligands near the active site of acetylcholinesterase mutant H287C: Progress on a new strategy for protection against organophosphate inactivation. *Proceedings of the VII International Meeting on Cholinesterases* in press.

Johnson, J.L., Cusack, B.M., Davies, M.P., Fauq A. and Rosenberry, T.L.. Substrate activation with a cationic acetanilide substrate in human acetylcholinesterase. *Proceedings of the VII International Meeting on Cholinesterases* in press.

ABSTRACTS

Johnson, J.L., Cusack, B., Davies, M.P., and Rosenberry, T.L. Ligand interactions within the active site of acetylcholinesterase. *VII International Meeting on Cholinesterases,* November, 2002

Rosenberry, T.L., Johnson, J. L., Cusack, B., Romanovskis, P. and Spatola, A.F. Cyclic inhibitors tethered near the acetylcholinesterase peripheral site. Progress on a new strategy for protection against organophosphate toxicity. *VII International Meeting on Cholinesterases,* November, 2002

Rosenberry, T. ., Johnson, J. L., Cusack, B., Romanovskis, P. and Spatola, A.F. Cyclic inhibitors tethered near the acetylcholinesterase peripheral site. Progress on a new strategy for protection against organophosphate toxicity. *Bioscience Medical Defense Review*, June, 2002

Cusack, B. M., Johnson, J.L., Hughes, T.F., McCullough, E.H., Romanovskis, P., Spatola, A.F., and Rosenberry, T.L. Modulation of acetylcholinesterase kinetics employing ligands tethered to the mutant H287C: A model for organophosphate inhibitor design. *Bioscience Medical Defense Review*, June, 2002

Johnson, J.L., Cusack, B., Davies, M.P., and Rosenberry, T.L. Ligand interactions within the active site of acetylcholinesterase. *Bioscience Medical Defense Review*, June, 2002

CONCLUSIONS

Our ultimate goal is to develop cyclic peptides that will effectively block access of organophosphates into the acylation site of AChE without restricting access of acetylcholine. We have developed a novel strategy that will restrict the location of cyclic peptides close to the P-site. Using the AChE mutant H287C we have developed a method to tether ligands to this site via MTS labeling. After a reductive methylation with [^{3}H]formaldehyde, the MTS tethered compound is linked to the enzyme via a disulfide linkage. Candidate compounds are synthesized with the MTS group that allows for covalent linkage to H287C AChE. However, we found that all of the enzyme was not modified. Using affinity chromatography on acridinium resin we were able to separate modified enzyme from unmodified enzyme. We take advantage of the separation differences on the affinity column which are based on the binding of the enzyme to the acridininum resin. That is, unmodified enzyme will bind to the column and elute with the competitive inhibitor, decamethanonium. Enzyme modified with ligands that interfer with the binding of the enzyme to the resin will either not be retained by the resin at all or elute in a NaCl wash. As proof of concept we used cationic trimethylammonium or acridinium ligands with demonstrated affinity for the AChE active site that are attached to MTS tethers of various lengths. H287C was labeled with 6 different MTS-derivatized compounds. (see Table I). Enzyme modified with compounds I and II behaved similar to unmodified and eluted with decamethonium. These compounds do not extend far enough into the P-site to restrict access to the resin. In contrast, enzyme modified with compounds IV, V, and VI eluted with the NaCl wash indicating decreased affinity for the resin. A comparison of relative catalytic activity per enzyme site (enzyme activity/dpm) showed a much lower ratio for the modified enzymes that eluted with NaCl than for the unmodified enzyme that eluted with decamethonium (see Figure 1B). A comparison of the dependence of hydrolysis rates on the substrate concentration demonstrated dramatic differences between unmodified and H287C modified with compounds II, IV and VI (Figure 2). Typical bell shaped profiles were observed with unmodified enzyme as well as enzyme modified with compound II. For enzymes modified with compounds IV and VI, the hydrolysis profiles were shifted to the right with no substrate inhibition apparent for compound VI. We examined the inhibition of enzyme activity by propidium and tacrine to demonstrate the extent that the tethers competed with access to the P-site or A-site. Using a modified Haldane equation which assumes two populations of AChE characterized by different K_I values, we compared K_{I2} values (Table 1). Modifications with I and II, the shortest tethers, had little effect on K_{I2} values. Enzymes modified with III, IV or V demonstrated reduced propidium inhibition, while modification with VI exhibited a dramatic decrease in the K_{I2}. For tacrine inhbition K_{I2} values increased only slightly with I-III and showed a 90-fold increase following modification with VI (Table 1).

Our plan is to use this strategy to evaluate cyclic peptides in which a lysine side chain is covalently linked to MTS. By using combinatorial libraries of peptides and the strategy we have proposed in this study, we hope to have results that will allow molecular modeling programs to generate more rigorous predictions of optimal peptide composition. Any possible candidate compounds would be stripped of the tethering constraint and tested against the wild type AChE.

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